CHAPTER 8. ISOLATION AND IDENTIFICATION OF *LISTERIA MONOCYTOGENES* FROM RED MEAT, POULTRY, EGG AND ENVIRONMENTAL SAMPLES (Revision 2; 11/08/99)

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8.1 Introduction

Listeria monocytogenes is a gram-positive rod-shaped bacterium associated with a variety of environments including soils, water, sewage, silage, as well as plant and animal food products. Although reported cases of human foodborne listeriosis are rare, the incidence of serious illness and death in affected individuals is high. Immunocompromised individuals, pregnant women, neonates and the elderly are particularly vulnerable.

Among all species in the genus *Listeria*, only *L. monocytogenes* has been implicated in human foodborne illness. The method described below employs well-established media and tests for the isolation and specific identification of β-hemolytic *L. monocytogenes*. The method is broadly applicable to raw and cooked ready-to-eat red meat and poultry products, including egg products. This protocol supercedes previous USDA/FSIS protocols for *L. monocytogenes*, including MLG Chapter 8, Revision 1 (1/12/99).

8.2 Equipment, Supplies, Media and Reagents

8.21 Equipment

a. Electronic top-loading balance capable of weighing a minimum of 25 ± 0.1 g (500 g capability recommended)
b. Stomacher™ (model 400 by Tekmar, or comparable model), OR blade-type blender, sterilized blender cutting assemblies and jars
c. Incubator, 30 ± 2°C
d. Incubator, 35 ± 2°C
e. Incubator, 20 or 25 ± 2°C
f. Vortex mixer
g. Phase-contrast microscope (40X and 100X objectives)
h. Fluorescent desk lamp or natural spectrum light source

8.22 Supplies

a. Supplies for all samples:

i. Sterile sample preparation supplies: scalpels, chisels, knives, scissors, spatulas, forceps, disposable or reusable dishes, pans or trays

ii. Stomacher® Strainer 400™ sterile filter bags or non-filtered bags or equivalent
iii. Non-filter plastic bags for double-bagging sample homogenates (optional)
iv. Pipets (e.g. 1 ml)
v. Pipettor and sterile disposable tips for dispensing 100 µl
vi. Microscope slides, cover slips and immersion oil
vii. Disposable plastic and/or platinum inoculating needles and loops
viii. Wax or waterproof ink marker
ix. Sterile cotton-tipped applicators (i.e. swabs)

b. Additional supplies necessary for environmental sampling:

i. 0.45-µm hydrophobic grid membrane filter system(e.g. SELeCT 56 mm/100-ml 0.45-µm Monitor, cat# BPM9261, BioPath Inc., 2611 Mercer Ave., W. Palm Beach, FL 33401, 800-645-2302, fax=407-655-3361 or equivalent)
ii. Vacuum flask/hose and vacuum source
iii. Sterile disposable scalpels
iv. Sterile forceps
v. Non-bactericidal sampling sponges (e.g. Nasco cat# B01299WA or equivalent)

8.23 Media

Refer to *Listeria* Media Appendix for Chapter 8, Revision 2 (11/08/99) for formulations and preparation instructions.

a. Media required for enrichment, plating and preliminary confirmation tests

i. Modified University of Vermont broth (UVM, also known as UVM1)
ii. Fraser broth (FB) or Fraser broth minus ferric ammonium citrate (FB-Fe)
iii. Modified Oxford agar (MOX)
iv. Horse blood overlay agar (HL, also known as HBO)
v. Trypticase soy agar with 5% sheep blood (TS-SBA, also known as CAMP test agar)
vi. Brain heart infusion broth (BHI broth)

b. Additional media for environmental samples

i. Dey-Engley (D/E) neutralizing broth (e.g. Nasco cat# B01256WA or equivalent)
ii. *Listeria* Repair Broth (LRB)

c. Optional media

i. Trypticase Soy Agar-Yeast Extract (TSA-YE)

8.24 Reagents and test systems

a. Reagents and materials required for routine tests

i. β-lysin CAMP factor discs (Remel #21-120) or equivalent.
ii. Biochemical test panel (MICRO-ID® *Listeria*, api®-*Listeria* system, or equivalent)

b. Reagents for supplemental tests

i. Ribosomal RNA-based test system (i.e. GenProbe Accuprobe® *L. monocytogenes*-specific test, GeneTrak® *L. monocytogenes*-specific test, or equivalent)
ii. Polyvalent *Listeria* antisera (Difco),types 1 and 4

8.25 Cultures

a. At least one *L. monocytogenes* positive control strain is required. Appropriate cultures include ATCC 19111, NCTC 7973 or other *L. monocytogenes* cultures validated to perform in an equivalent manner.

b. At least one *L. innocua* negative control culture is required. Appropriate cultures include ATCC 33090 or other *L. innocua* validated to perform in an equivalent manner.

c. Other *Listeria* spp., such as *L. seeligeri, L. grayi* and *L. ivanovii*, may be necessary as supplemental negative controls for some tests in some circumstances.

d. If the β-lysin CAMP factor test is not employed, *Staphylococcus aureus* ATCC 25923 or ATCC 49444, and *Rhodococcus equi* ATCC 6939 are required to perform the traditional CAMP test.

8.3 Detection and isolation procedures

8.31 Sample preparation (DAY 1 or prior)

a. Meat, poultry and egg products: A 25 ± 1.0-g portion is used for raw and processed red meat, poultry and egg product testing. The test portion should be a composite representative of the entire sample or available samples common to a specific lot.

i. Intact retail packages must be disinfected at the incision sites immediately prior to incision for sampling. Appropriate disinfectants include but are not limited to ca. 3% hydrogen peroxide, ca. 70% ethanol or ca. 70% isopropanol. If the package does not appear to be clean, scrub gently using soapy water and rinse thoroughly prior to disinfection. A sterile scalpel may be helpful for cutting the packaging. Aseptically pull the packaging away to expose the product for sampling.

ii. Using a sterile scalpel, knife, spoon, chisel or other tool, cut small pieces from representative sites on the sample and/or a variety of same-lot samples to prepare a composite sample.

iii. Place 25 ± 1.0-g of the composite sample in a sterile Stomacher™ bag (i.e. filter bag recommended). If necessary, double-bag with a non-filter bag prior to stomaching (e.g. zip-lock bag).

iv. If analysis of the test portion is not to be initiated within 1 h, store at –20 to -30°C. Do not dilute the sample until ready to initiate analysis.

v. For analysis, proceed to Section 8.32.

b. Outbreak/recall samples: Some samples or sample lots, particularly those implicated in foodborne illness outbreaks, may require analysis of up to thirteen 25-g test portions. The test portion should be a composite representative of the entire sample or available samples common to a specific lot. The need for multiple subsample analyses must be determined on a case-by-case basis.

8.32 Primary enrichment in UVM broth (DAY 1, REQUIRED)

Refer to **Diagram 1** for a flow diagram of the *L. monocytogenes* procedure from enrichment to confirmation.

a. For all meat, poultry and egg product samples (including outbreak/recall samples):

To the 25 ± 1.0 g test portion, dispense 225 ± 5 ml (or 225 ± 5 g) of UVM broth. Stomach or blend for 2 ± 0.2 minutes. If blended, aseptically transfer the homogenate to an appropriate sterile container. Incubate the homogenate at 30 ± 2°C for 22 ± 2 h. Proceed to Section 8.33.

b. For environmental sponge samples:

For sampling: Prior to sampling and shipment to the laboratory, add 10 ± 1 ml of Dey-Engley Neutralization Broth (D/E) to the bagged sponge and allow complete absorption. After sampling, re-bag the sponge in a sterile Whirl-Pak® bag or equivalent. If enrichment in both UVM and LRB is desired, collect two sponge samples per site. Ship sponge samples cold to the laboratory by an overnight service.

For laboratory analysis: Add 200 ± 5 ml of UVM broth to each bagged sponge sample and stomach 2 ± 0.2 min. Optionally, a second sample representing the same or similar environmental area can be enriched using LRB broth. If LRB is to be employed, add 200 ± 5 ml of LRB broth to the second bagged sponge sample and stomach 2 ± 0.2 min.

Incubate at 30 ± 2°C for 22 ± 2 h. Proceed to Section 8.33.

NOTE: The use of LRB for duplicate samples may, in some circumstances, encourage the growth of *L. monocytogenes* injured due to exposure to sanitizer residues on environmental surfaces (Sallam and Donnelly, 1991).

c. For environmental aqueous chilling solutions and surface rinse solutions:

Aqueous chilling solutions may include water, brine and propylene glycol solutions.

i. Filter an appropriate volume (100 ± 2 ml recommended if available) of sample solution through one or more 0.45-μm hydrophobic grid membrane filters. These filters can be easily clogged by particulates. Therefore, more than one may be necessary to filter the entire test portion. Pre-sieving may remove a significant number of *Listeria* cells from the sample and should be applied only if absolutely necessary.

ii. Using sterile forceps, aseptically remove the membrane from the plastic housing and transfer to a stomacher bag. A sterile scalpel may be used to cut around the circumference of the hydrophobic grid membrane to excise it from the filter housing (e.g. SELeCT® system). All membranes associated with a given sample may be combined in a single enrichment bag.

iii. Add 200 ± 5 ml or additional volume of UVM broth sufficient to completely cover the filters. Stomach 2 ± 0.2 minutes. Incubate the homogenate at 30 ± 2°C for 22 ± 2 h. Proceed to Section 8.33.

8.33 Secondary enrichment in FB and direct plating of UVM (DAY 2, REQUIRED)

a. Transfer 0.1 ± 0.02 ml of the UVM enrichment to 10 ± 0.5 ml of FB. As per media preparation instructions, be sure that appropriate supplements have been added to the FB prior to inoculation. Incubate inoculated FB tubes at 35 ± 2°C for 26 ± 2 h.

If an alternative screening test is to be employed, FB-Fe (i.e. FB without ferric ammonium citrate) may be used instead of FB (Section 8.35). Incubate the FB-Fe or FB for the period specified by the alternative screening test system manufacturer (refer to Section 8.35) but no less than 20 h.

b. Aseptically dip a sterile cotton-tipped applicator or

equivalent into the UVM and swab 25-50% of the surface of a MOX plate. Use a loop to streak for isolation from the swabbed area onto the remainder of the plate. Incubate the MOX at 35 $\pm$ 2°C for a minimum of 24 h.

c. Proceed to Section 8.34.

NOTE: Direct plating of UVM should be performed regardless of whether an alternative screening procedure is to be applied.

8.34 Examination of UVM-streaked MOX and interpretation/plating of 26-h FB (DAY 3, REQUIRED)

a. Examine the UVM-streaked MOX for colonies with morphology typical of *Listeria* spp. At ca. 24 h, suspect colonies are typically small (ca. 1 mm) and are surrounded by a zone of darkening due to esculin hydrolysis.

i. If suspect colonies are present on MOX, transfer suspect colonies to HL agar as described in Section 8.37. Then, proceed to step b below.

ii. If no suspect colonies are evident, re-incubate the MOX plate until a total incubation time of 48 $\pm$ 2 h has been achieved. If an alternative screening procedure has been employed (Section 8.35), refer to Step 8.35-c to determine the appropriate interpretation of combined MOX and screen test results.

b. After overnight incubation, FB can be examined for the potential presence of *L. monocytogenes* either by visual examination of the broth for esculin hydrolysis (i.e. darkening of the broth) or by application of an alternative screening method (i.e. immunoassay, nucleic acid-based assay, etc.).

i. For instructions on the potential applicability and use of an alternative screening method, proceed to Section 8.35.

ii. If an alternative screening method is **not** to be applied, examine the 10 ± 0.5-ml FB aliquot after 26 ± 2 h of incubation for darkening due to esculin hydrolysis.

1. If any degree of FB darkening is evident, aseptically dispense 100 ± 20 µl of the FB onto a MOX plate. Swab or streak 25-40% of the surface of the MOX plate with the FB inoculum. Use a loop to streak for isolation from the initial swab/streak quadrant onto the remainder of the plate. Incubate the MOX plate at 35 ± 2°C for a minimum of 24 h. Proceed to Section 8.36.

2. If no FB darkening is evident, re-incubate the FB at 35 ± 2°C for an additional 17-25 h until a total incubation time of 48 ± 2 h has been achieved. Proceed to Section 8.36.

8.35 Alternative rapid screening procedures (DAY 3, OPTIONAL)

Under some circumstances, an alternative screening test system (e.g. immunoassay, nucleic acid-based assay or other "rapid" method) may be employed instead of FB darkening for determining the potential presence of *L. monocytogenes*. The use of such a system would reduce the turnaround time required for determining most negative samples by one day. An alternative screening procedure may be considered acceptable for this application if the test system has been validated by a multi-laboratory study to meet the following conditions:

a. The validation study must determine that the alternative screen test is equivalent or superior to the FB darkening test specified in Sections 8.34 and 8.36.

b. It must be validated using appropriate sample matrices.

c. The test system must be validated for specific use on Day 3 (i.e. from overnight FB) using the UVM/FB enrichment scheme delineated in this protocol. The alternative test must be applied on Day 3 to all FB. Sample preparation procedures and incubation temperature/period parameters for primary enrichment in UVM must not be altered.

d. FB must be prepared, inoculated and incubated as

specified in this protocol, with the following exceptions:

i. Darkening of FB can interfere with the performance and interpretation of many alternative screening test systems. Therefore, FB that does **not** contain ferric ammonium citrate (i.e. FB-Fe; refer to *Listeria* Media Appendix) is recommended for this application. Keep in mind that FB-Fe is **not** functional for visual examination of esculin hydrolysis (i.e. darkening).

ii. Continued incubation of FB-Fe beyond the period required for the alternative screen test system is not necessary.

iii. Total incubation time of FB-Fe or FB can be reduced to a minimum of 20 h to accommodate an appropriately validated alternative screening procedure.

The use of an alternative screening procedure does not preclude the need for examining the UVM-streaked MOX plate at 24 $\pm$ 2 h. If the alternative screening test is negative, it is **not** necessary to re-incubate and re-examine the MOX plate at 48 $\pm$ 2 h. **Both** the alternative screen test result **and** the UVM-streaked MOX plate result must be considered to determine the disposition of the sample as follows:

a. **Screen (+) MOX (+):** Proceed with follow-up of suspect UVM-streaked MOX colonies (i.e. subculture to HL plate as per Section 8.37). Subculture FB-Fe or FB to MOX (Step 8.34-b-ii-1), incubate and follow-up.

b. **Screen (+) MOX (-):** Subculture FB-Fe or FB to MOX (Step 8.34-b-ii-1), incubate and follow-up. Re-incubate and follow-up UVM-streaked MOX (Steps 8.34-a-ii and 8.36-a).

c. **Screen (–) MOX (+):** Proceed with follow-up of suspect MOX colonies (i.e. subculture to HL plate as per Section 8.37).

d. **Screen (–) MOX (-):** Report the sample as *L. monocytogenes* negative.

NOTE: USDA/FSIS does not promote, recommend or guarantee the applicability of any specific commercial test system proposed for the above use.

8.36 Interpretation of 48-h FB and other follow-up (DAY 4, REQUIRED)

a. Examine and select suspect colonies from any MOX agar plate pending analysis (i.e. MOX plates streaked from 26 ± 2 h FB and/or UVM) as described in Section 8.33.

b. If an alternative screening test was not employed and non-darkened 26 ± 2 h FB was re-incubated, re-examine the FB for evidence of darkening after 48 ± 2 h of total incubation.

i. If any degree of darkening is evident, swab, streak and incubate a MOX plate as described in Section 8.33.

ii. If no darkening of FB is evident and no suspect MOX and/or HL colonies have been demonstrated, the sample is considered negative for *L. monocytogenes*.

8.37 Isolation and purification procedures (AS EARLY AS DAY 3 OR AS LATE AS DAY 6, REQUIRED)

a. If suspect colonies are present on MOX from any source, use a loop or equivalent sterile device to contact **a minimum of 20** of the suspect colonies and collectively streak for isolation on one or more HL agar plates. Alternatively, a swipe of suspect growth representing at least 20 colonies may be used. Incubate the streaked HL at 35 ± 2°C for 19 ± 3 h.

b. After 19 ± 3 h of incubation, examine the HL plate(s) against back light for translucent colonies surrounded by a small zone of β-hemolysis (i.e. clearing of the blood).

i. If at least one suspect colony is clearly isolated (i.e. appears pure), proceed to confirmatory testing (Section 8.4 below). Hold all HL plates containing suspect colonies (room temperature or refrigeration) until confirmatory testing is complete.

NOTE: In samples containing mixed populations of *Listeria* spp., *L. innocua* can easily overgrow *L. monocytogenes* as enrichment progresses (Petran and Swanson, 1992, and Curiale and Lewus,1994) and confound isolation from FB. Therefore, if the suspect HL colony or colonies have been generated from follow-up of a UVM-streaked MOX plate, there is a high probability of confirmation compared to follow-up of *Listeria* spp. from extended enrichment in FB.

ii. If suspect colonies or β-hemolytic growth are present on HL but not clearly isolated, re-streak representative suspect colonies/growth onto one or more fresh HL plates and incubate as before.

NOTE: Isolated MOX colonies cannot be assumed to be pure due to the highly selective nature of the medium. Therefore, MOX colonies must be purified on HL prior to inoculation of confirmatory test media.

iii. If no suspect isolates are present on HL, pursue follow-up of MOX and/or HL isolates from other branches of analysis (e.g. FB follow-up vs. UVM direct streak follow-up). If no branch of the analysis produces suspect β-hemolytic colonies on HL, the sample may be reported as negative for *L. monocytogenes*.

8.4 Confirmation and other identification procedures

Confirmatory identification of *L. monocytogenes* consists of preliminary confirmation tests (Section 8.41) followed by biochemical tests (Section 8.42). The CAMP test (Section 8.43) and genetic tests (Section 8.44) may be required in certain circumstances. All confirmatory identification tests require a pure culture (Section 8.37). Refer to the attached **Diagram 2** for a flow diagram of confirmatory procedures.

8.41 Preliminary confirmation tests for *Listeria* spp.

a. Inoculation of preliminary confirmation test media (REQUIRED)

Use **one** isolated HL colony only to inoculate, in order, a BHI broth aliquot and (optionally) a fresh HL plate to confirm purity. In addition, media required for inoculation of biochemical test systems (e.g. HL, BHI agar, TSA-YE, TSA-SBA or equivalent as described in Section 8.42) must be inoculated from the **same** colony or growth subcultured from that colony. A minimum of one colony must be confirmed. If the first selected suspect HL colony does not confirm as *L. monocytogenes*, confirmation must be attempted for additional suspect HL colonies, if available, until at least three isolates from the test portion have failed confirmation.

NOTE: Despite the fact that all suspect colonies on HL may have an identical morphology, it is critical that all confirmatory tests are performed on growth directly from or subcultured from a single HL colony. This will guarantee that all tests are performed on a single clone and that the integrity of that clone can be maintained for subtyping analyses.

Incubate BHI broth at 18-25◦C for 16-18 h. Proceed to step 8.41 c.

Incubate the HL purity streak plate at 35 $\pm$ 1◦C for 20 $\pm$ 2 h. Proceed to Section 8.42.

b. Purity streak test (OPTIONAL IF SELECTED HL COLONY APPEARS PURE)

Examine the re-streaked 20 $\pm$ 2-h HL plate for consistent morphological characteristics. The colonies on this second HL plate should represent a single clone. If the culture appears to be mixed, repeat Steps 8.37 b-ii and/or 8.41 a. A **pure culture is required** for all confirmatory tests.

c. Tumbling motility test (REQUIRED)

After 16-18 h of incubation of BHI broth at 18-25◦C, prepare a wet-mount (i.e. small drop of culture sandwiched between a slide and cover slip). Using the 100X oil immersion objective (phase contrast microscopy recommended), examine the wet-mount culture for small rods that exhibit an active end-over-end tumbling/rotating movement characteristic of *Listeria* spp.

i. If cell morphology and motility are not characteristic of *Listeria* spp., and the culture appears pure, report the sample as negative for *L. monocytogenes*.

ii. If a mixture of typical *Listeria* cells and cells that do not have the characteristic *Listeria* morphology (i.e. small rods) are present, streak a loopful of the contaminated BHI broth onto fresh HL agar for further purification (Step 8.37 b-ii).

iii. If no growth is evident at 16-18 h, re-incubate at 18-25◦C until growth is evident or up to a total of 48 h.

iv. If cell morphology is typical, tumbling motility is evident and the culture appears pure, proceed with biochemical confirmation (Section 8.42 below).

NOTE: The 18-25◦C incubation temperature for BHI broth is critical for demonstrating tumbling motility. *Listeria* spp. may produce fewer or no flagella at higher incubation temperatures.

8.42 Biochemical tests (REQUIRED)

If the suspect culture is pure and demonstrates a cell morphology and tumbling motility characteristic of *Listeria* spp., confirmatory biochemical tests must be performed. Either of two commercially available test systems (MICRO-ID® *Listeria* or api®-*Listeria*) or validated equivalent systems, including well-established schemes involving traditional tube biochemical media (e.g. Compendium of Methods for the Microbiological Examination of Foods, Bacteriological Analytical Manual), may be employed.

a. MICRO-ID® *Listeria* test system

i. Follow the instructions provided by the manufacturer for inoculation and interpretation of the test panel.

ii. TSA-YE, BHI, TSA-SBA or HL agar inoculated and incubated as described in Section 8.41 may be used for preparing the inoculum suspension. All growth on the agar of choice must represent the same clone (i.e. be a pure culture). Therefore, do not use an HL plate inoculated from a mixed culture (e.g. from MOX).

iii. A CAMP test (Section 8.43) must be performed to augment MICRO-ID® results.

iv. At minimum, one *L. monocytogenes* positive control must be analyzed concurrently with sample isolates. Each lot of MICRO-ID® kits received must be tested for proper performance using *L. monocytogenes*, *L. innocua* and *L. seeligeri* control cultures.

Exercise caution in interpreting the identification of atypical *Listeria* spp. isolates when using the MICRO-ID® *Listeria* system. Be aware that rhamnose-negative *L. monocytogenes* do exist (Wiedmann *et al.*, 1997) as well as β-hemolytic *L. innocua*. Therefore, all β-hemolytic CAMP-positive *Listeria* spp. identified as *L. innocua* by MICRO-ID® must be analyzed using a ribosomal RNA-based test system as described in Section 8.44-a.

b. api®-*Listeria* test system

i. Follow the instructions provided by the manufacturer for inoculation and interpretation of the test panel.

ii. TSA-YE, BHI, TSA-SBA or HL agar may be used for preparing the inoculum suspension. All growth on the agar of choice must represent the same clone (i.e. be a pure culture). Therefore, do not use an HL plate inoculated from a mixed culture (e.g. from MOX).

iii. Cultures identified by api®-*Listeria* as *"L. monocytogenes/innocua"*(i.e. as opposed to either individual identity) must be further characterized using a ribosomal RNA-based test system as described in Section 8.44-a.

iv. At minimum, one *L. monocytogenes* positive control must be analyzed concurrently with sample isolates. Each lot of api®-*Listeria* kits received must be tested for proper performance using *L. monocytogenes*, *L. innocua* and *L. seeligeri* control cultures.

8.42 CAMP test (REQUIRED TO AUGMENT BIOCHEMICAL TESTING)

The CAMP test is required to augment traditional biochemical or MICRO-ID® test results, or to resolve the hemolytic capability of indeterminate strains. Either of two test options may be employed:

a. β-lysin CAMP factor test

This test system may provide results that are easy to interpret compared to the traditional CAMP test. Therefore, the β-lysin CAMP factor test is recommended over the traditional test.

i. Aseptically place (i.e. with sterile forceps) a β-lysin disc in the approximate center of a TS-SBA plate. A 9 ± 1-ml TS-SBA plate is easier to interpret than thicker plates and is recommended.

ii. Individually and aseptically streak four to eight isolates each as straight lines radiating away from the disc. The inoculation line should almost but not quite touch the disc. Be sure to include positive and negative control cultures. A non-hemolytic *L. innocua* is an appropriate negative control.

iii. Incubate at 35 ± 2°C for 24 ± 2 h.

iv. An arrowhead-shaped zone of β-hemolysis (i.e. clearing of the blood) surrounding the inoculum line proximal to the disc indicates a positive CAMP reaction. *L. monocytogenes, L. seeligeri* and *L. ivanovii* are CAMP positive by this test. However, *L. ivanovii* demonstrates relatively intense β-hemolysis distal to the disk and, therefore, can be distinguished from the other two species. Non-hemolytic *Listeria* spp. are CAMP negative.

v. If a suspected β-hemolytic *Listeria* spp. does not produce a CAMP-positive reaction with either reference culture at 24 ± 2 h, continue to incubate the culture at 35 ± 2°C until a total incubation time of 48 ± 2 h has been achieved. Re-examine as before. If a CAMP-positive reaction is still not evident at 48 ± 2 h, ribosomal RNA-based testing is required to determine the genetic identity of the isolate (Section 8.44 a).

b. Traditional CAMP test

If the CAMP test is necessary and β-lysin discs are not available, perform the following test:

i. For the traditional culture CAMP test, apply single-line streaks of *S. aureus* (i.e. ATCC 4944 or ATCC 25923) and *R. equi* (i.e. ATCC 6939) reference cultures on a TS-SBA plate in parallel and 3-4 cm apart.

ii. Streak test cultures between and perpendicular to the two reference cultures (i.e. like rungs of a ladder). The test culture streak must be 2-4 mm from each reference culture streak. Test and reference cultures must not touch or be cross-contaminated in any manner.

iii. Incubate 24 $\pm$ 2 h at 35°C.

iv. Examine the test culture streaks for enhanced β-hemolysis (i.e. clearing of the blood) at both ends proximal to the reference cultures. The zone of enhanced β-hemolysis may resemble an arrowhead, circle or rectangle. The presence of this zone indicates a CAMP-positive reaction. Absence of enhanced β-hemolysis indicates a CAMP-negative reaction. *L. monocytogenes* and *L. seeligeri* are CAMP-positive to the *S. aureus* reference strain and CAMP-negative to *R. equi*. In contrast, *L. ivanovii* is CAMP-positive to the *R. equi* reference strain and CAMP-negative to the *S. aureus* strains.

v. If a suspected β-hemolytic *Listeria* spp. does not produce a CAMP-positive reaction with either reference culture at 24 $\pm$ 2 h, continue to incubate the culture at 35 $\pm$ 2°C until a total incubation time of 48 $\pm$ 2 h has been achieved. Re-examine as before.

vi. If the culture does not produce a CAMP-positive reaction with the *S. aureus* culture, but has or does clearly demonstrate β-hemolysis on HL agar, ribosomal RNA-based testing is required to determine the genetic identity of the isolate (Section 8.44 a).

8.44 Genetic identification tests

a. Ribosomal RNA-based tests (REQUIRED FOR SUSPECTED ATYPICAL *L. MONOCYTOGENES*)

A ribosomal RNA-based test is required to resolve the identity of any suspected atypical *L. monocytogenes* strain. In some circumstances, available phenotypic tests cannot clearly distinguish strains of *L. monocytogenes* from *L. innocua*. In particular, rhamnose-negative β-hemolytic *L. monocytogenes* can be misidentified as *L. innocua*. The following *L. monocytogenes*-specific ribosomal RNA-based tests are commercially available:

i. GenProbe AccuProbe® *L. monocytogenes*-specific test or equivalent

ii. GENE-TRAK® *L. monocytogenes*-specific test or equivalent

Follow the test kit instructions provided by the manufacturer for performing and interpreting these tests.

An *L. monocytogenes* positive control, *L. innocua* negative control and uninoculated control must be analyzed concurrently with sample isolates. Periodic testing of other *Listeria* spp. (e.g. *L. seeligeri, L. grayi*) is recommended.

A positive ribosomal RNA-based test result from either of the above test systems indicates that atypical phenotypes are confirmed *L. monocytogenes*. A negative result indicates that atypical phenotypes are not *L. monocytogenes*.

b. Pulsed-field gel electrophoresis (PFGE)

PFGE is a highly discriminative and reliable means of subtyping *L. monocytogenes* strains. The pulsotype (i.e. “genetic fingerprint”) derived from PFGE analysis is used in tandem with epidemiologic evidence to link clinical and food isolates implicated in foodborne illness. Standardized PFGE methodology (Graves, 1998) and internet-based exchange of federal and state laboratory pulsotype data (i.e. PulseNet) facilitate epidemiologic and traceback investigations.

8.45 Serological tests (OPTIONAL)

Serological testing may be employed to further characterize isolates already confirmed to be *L. monocytogenes*. *Listeria* O Type (i.e. somatic antigen) "1", "4" and "poly" antisera are available from Difco Laboratories (Becton-Dickinson and Co.). The use of these antisera is described in the Difco Manual, 11th Edition, pp.648-651.

8.5 Enumeration procedures

The estimated number of *L. monocytogenes* per gram for a given sample can be determined by either of two enumeration methods:

8.51 Most Probable Number (MPN) enumeration method (RECOMMENDED FOR ENUMERATION)

The MPN method is the most sensitive of the two enumeration method options and must be used for determining counts of samples anticipated to be contaminated with 100 cfu *L. monocytogenes*/g or less. The MPN method generally requires more resources than the direct plating enumeration method.

a. Stomach or blend 25 $\pm$ 1 g of sample with 225 ml of UVM as specified in Section 8.32.

b. Dispense 10 $\pm$ 0.5 ml of the undiluted UVM/sample homogenate into each of three sterile culture tubes. Each tube represents a 1.0-g test portion.

c. Dispense 1 $\pm$ 0.05 ml of the undiluted UVM/sample homogenate into each of three tubes containing 9-10 ml of UVM broth. Each tube represents a 0.1-g test portion.

d. Dispense 0.1 $\pm$ 0.005 ml (i.e. 100 $\pm$ 5 μl) of the undiluted UVM/sample homogenate into each of three tubes containing 9-10 ml of UVM broth. Each tube represents a 0.01-g test portion.

e. If an additional dilution is necessary, prepare two successive ca. 1:10 dilutions (i.e. total dilution of ca. 1:100) of the undiluted UVM/sample homogenate in fresh UVM. Dispense 1 $\pm$ 0.05 ml of the second dilution (1:100) into a 9 $\pm$ 0.5 ml aliquot of UVM. Repeat for two additional tubes representing a 0.001-g test portion. For additional dilutions, prepare additional ca. 1:10 dilutions of the ca. 1:100 dilution above and dispense as before.

f. Incubate all nine UVM tubes and the remainder of the undiluted UVM/sample homogenate (i.e. the qualitative test) as specified in Section 8.32. Perform isolation and identification of *L. monocytogenes* for each of the ten individual test aliquots as specified in Sections 8.33 through 8.4. The following procedures may be employed to simplify and expedite analysis:

i. Direct streaking of UVM MPN tubes to MOX agar (Section 8.33) may not be necessary under some circumstances. If qualitative analysis was performed on a given sample prior to enumeration, and direct plating of UVM to MOX was necessary for isolation of *L. monocytogenes*, direct plating of UVM is required for all MPN tubes. If qualitative analyses have not been performed prior to enumeration, direct plating is required for the concurrent qualitative analysis only and is optional for the UVM MPN tubes. However, if direct streaking of UVM is ultimately found to be necessary for isolation of *L. monocytogenes* from the sample, the sample should be re-enumerated by MPN and direct streaking must be performed from all UVM MPN tubes.

ii. An alternative screen test meeting the criteria described in Section 8.35 may be applied to determine FB/FB-Fe tubes that are negative from those that are potentially positive and require follow-up.

iii. As with the qualitative procedure, at least one colony must be confirmed from three available suspect isolates on the HL plate(s) representing **each** positive FB in the MPN series, including that from the undiluted UVM/sample homogenate. A ribosomal RNA-based test (Section 8.44) may be employed to supplant biochemical/CAMP testing and

expedite confirmation of **most** isolates. However, both biochemical/CAMP testing and ribosomal RNA-based testing must be used in tandem to characterize **at least one** suspect isolate representing the highest dilution found to contain suspects.

g. Record the number of tubes per dilution that were ultimately found to be *L. monocytogenes* positive. Use a 3-tube MPN table (e.g. FSIS Microbiology Laboratory Guidebook) to determine the estimated number of *L. monocytogenes* per gram of product. If all tubes are negative but the undiluted UVM/sample homogenate (i.e. the qualitative test) is positive, report the sample as positive at an enumerated level less than the lowest value on the table.

8.52 Direct plating enumeration method

In some circumstances, direct plating of the UVM homogenate onto MOX agar can be used to estimate the number of *L. monocytogenes* per gram of sample. Direct plating generally requires fewer resources than the MPN method and may expedite quantitative analysis in some circumstances. However, MOX agar can inhibit the growth of non-enriched *L. monocytogenes*, particularly sublethally injured cells. As a result, the direct plating method is considerably less sensitive than the MPN method and may underestimate the actual number of *L. monocytogenes* in a sample test portion. The direct plating method should be applied only to the analysis of samples anticipated to contain high levels of *L. monocytogenes* (i.e. 100 cfu/g or greater).

a. Stomach or blend 25 $\pm$ 1.0 g of sample with 225 $\pm$ 5 ml of UVM as specified in Section 8.32.

b. Dispense and evenly distribute (i.e. use a “hockey stick” or equivalent) 0.1 $\pm$ 0.005 ml (100 $\pm$ 5 μl) of the UVM homogenate onto each of **ten** MOX plates. The **sum** of the count of these plates represents a 0.1-g test portion.

c. Dispense and evenly distribute 0.1 $\pm$ 0.005 ml (100 $\pm$ 5 μl) of the UVM/sample homogenate onto each of **two** MOX plates. The **average** count of these two plates represents a 0.01-g test portion.

d. Using 10 $\pm$ 0.2-ml aliquots of UVM, prepare at least two successive ca. 1:10 dilutions of the UVM/sample homogenate. Dispense and distribute onto duplicate MOX plates as described above. The **average** counts for each plate pair for these two dilutions represents the 0.001-g and 0.0001-g test portions.

e. Incubate all MOX plates as described in Sections 8.34 a and b.

f. Count the suspect colonies (i.e. surrounded by dark zone) and calculate an average per plate for a given plate dilution pair.

g. Purify and confirm a minimum of **ten colonies per plate dilution** as specified in Sections 8.37 through 8.4. As with the qualitative procedure, at least one colony must be confirmed from three available suspect isolates on the HL plate(s) representing ten suspect colonies on the MOX dilution pair. A ribosomal RNA-based test (Section 8.44) may be employed to supplant biochemical/CAMP testing and expedite confirmation of **most** isolates. However, both biochemical/CAMP testing and ribosomal RNA-based testing must be used in tandem to characterize **at least one suspect** isolate representing the **highest MOX plate dilution** found to contain suspects.

h. For the 0.1-g test portion, multiply the number of MOX colonies confirmed to be *L. monocytogenes*-positive by the dilution factor and the **total number** of equivalent colonies **on all ten plates** to determine the estimated count. For the 0.01-g and more dilute test portions, multiply the number of MOX colonies confirmed to be *L. monocytogenes*-positive by the dilution factor and the **average number** of equivalent colonies per plate to determine the estimated count. The count from the dilution found to contain 25-250 *L. monocytogenes* colonies, if available, should be used for estimating the count per gram of sample. Successive dilutions found to be within this range should be averaged.

8.6 Quality control procedures

The correct performance of all stages of the analysis, including enrichment, screening tests, plating and all confirmatory tests, must be verified through the use of appropriate controls.

a. For enrichment, screening, plating and verification of tumbling motility, control cultures must be transferred from one medium to the next in the sequence of analysis used for the samples. For these stages of analysis, the following requirements apply:

   i. If FB darkening or an alternative screen test for *Listeria* spp. is employed, one *L. monocytogenes* positive control and one uninoculated media control are required for each set of concurrently analyzed samples.

   ii. If an *L. monocytogenes*-specific alternative screen test is employed, one *L. monocytogenes* positive control, one *L. innocua* negative control and one uninoculated media control are required for each set of concurrently analyzed samples.

   iii. For the uninoculated control, use a 225-ml aliquot of UVM broth. For all subsequent uninoculated control tests, use one unit of the medium at the volume specified for the test. Investigate the source of any contaminating organisms.

b. Specific control requirements for each confirmatory test are addressed in the appropriate sections of this protocol.

c. Additional (i.e. secondary) control cultures may be employed for individual tests or the entire sequence of analysis at the discretion of the laboratory.

d. Every lot or batch of media should be tested with at least one culture of *L. monocytogenes*. Every lot or batch of HL agar must be tested with at least one *L. monocytogenes* positive control and a non-hemolytic *L. innocua* negative control.

e. *L. monocytogenes* and *L. innocua* control culture inocula are prepared and used as deemed appropriate for the media to be tested. Ideally, an empirically determined standardized quantity of inoculum should be employed.

8.7 Safety procedures

a. Laboratory personnel must abide by CDC guidelines for manipulating Biosafety Class II pathogens. A Class II laminar flow biosafety cabinet is recommended for activities with potential for aerosolization of pathogens.

b. Pregnant women and potentially immunocompromised individuals must be prohibited from laboratory rooms or areas where *L. monocytogenes* isolation or identification procedures are in progress. Although a properly sanitized laboratory area should not harbor *L. monocytogenes* or other pathogens, supervisors should use their own discretion in allowing high-risk individuals into these areas when not in use for these activities.

8.8 Culture storage and shipment procedures

a. BHI or TSA-YE slants may be used for short-term storage of *Listeria* spp. The culture should be stabbed into the agar using an inoculating needle. Tubes should be sealed with Parafilm® or equivalent to prevent desiccation and stored at 4 $\pm$ 2°C. Under these conditions, *Listeria* spp. can remain viable for many months.

b. For long-term storage (i.e. for more than one year) or to assure that the genetic character of the strain does not change over time (e.g. lose plasmids or other unstable genetic elements), cultures should be lyophilized and/or frozen at –20 to -80°C. Fetal calf serum or commercially available cryobead products are appropriate media for frozen storage of *Listeria* spp.

8.9 References

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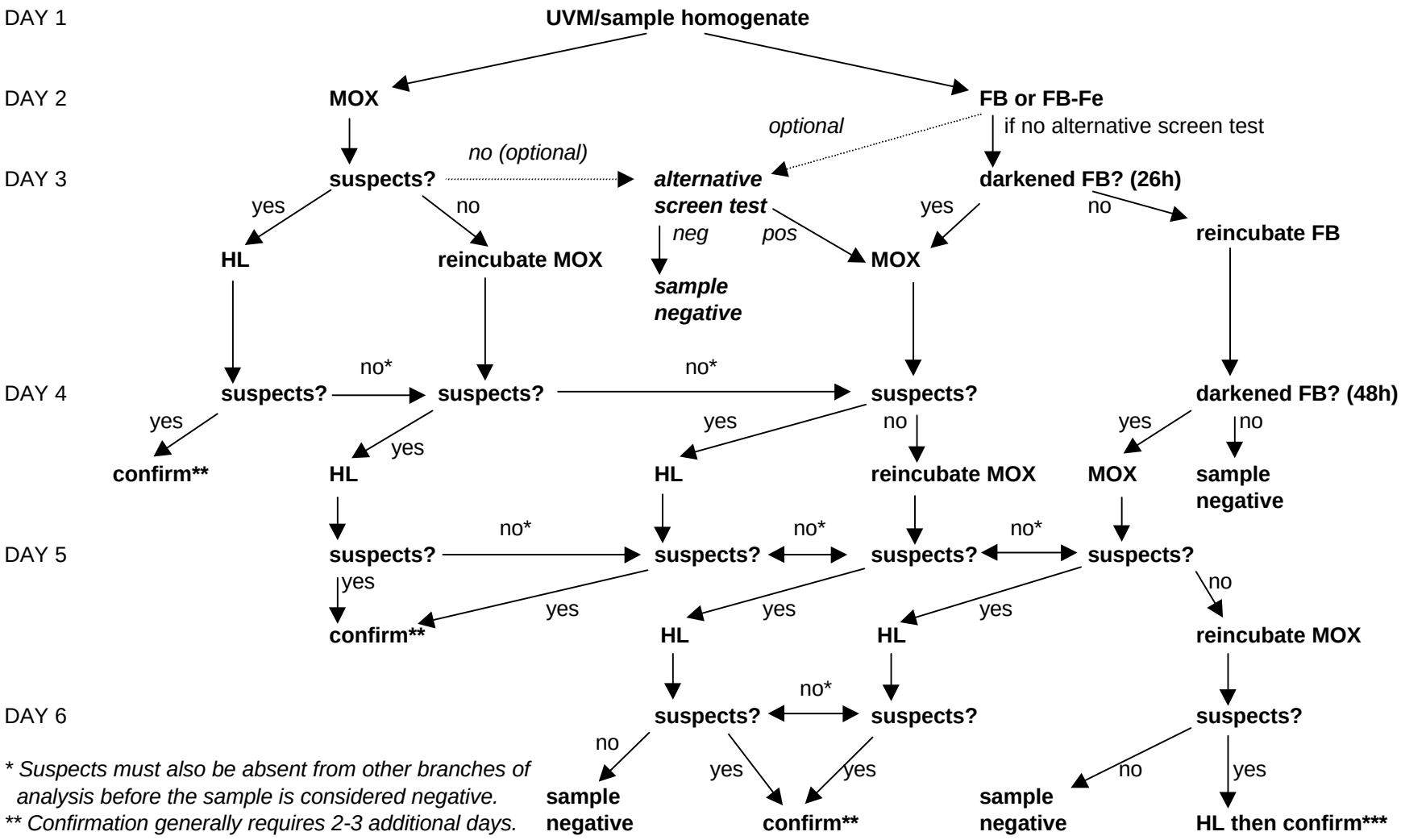
Diagram 1 **Flow diagram for *Listeria monocytogenes* analysis, MLG Chapter 8, Revision 2 (11/08/99)**

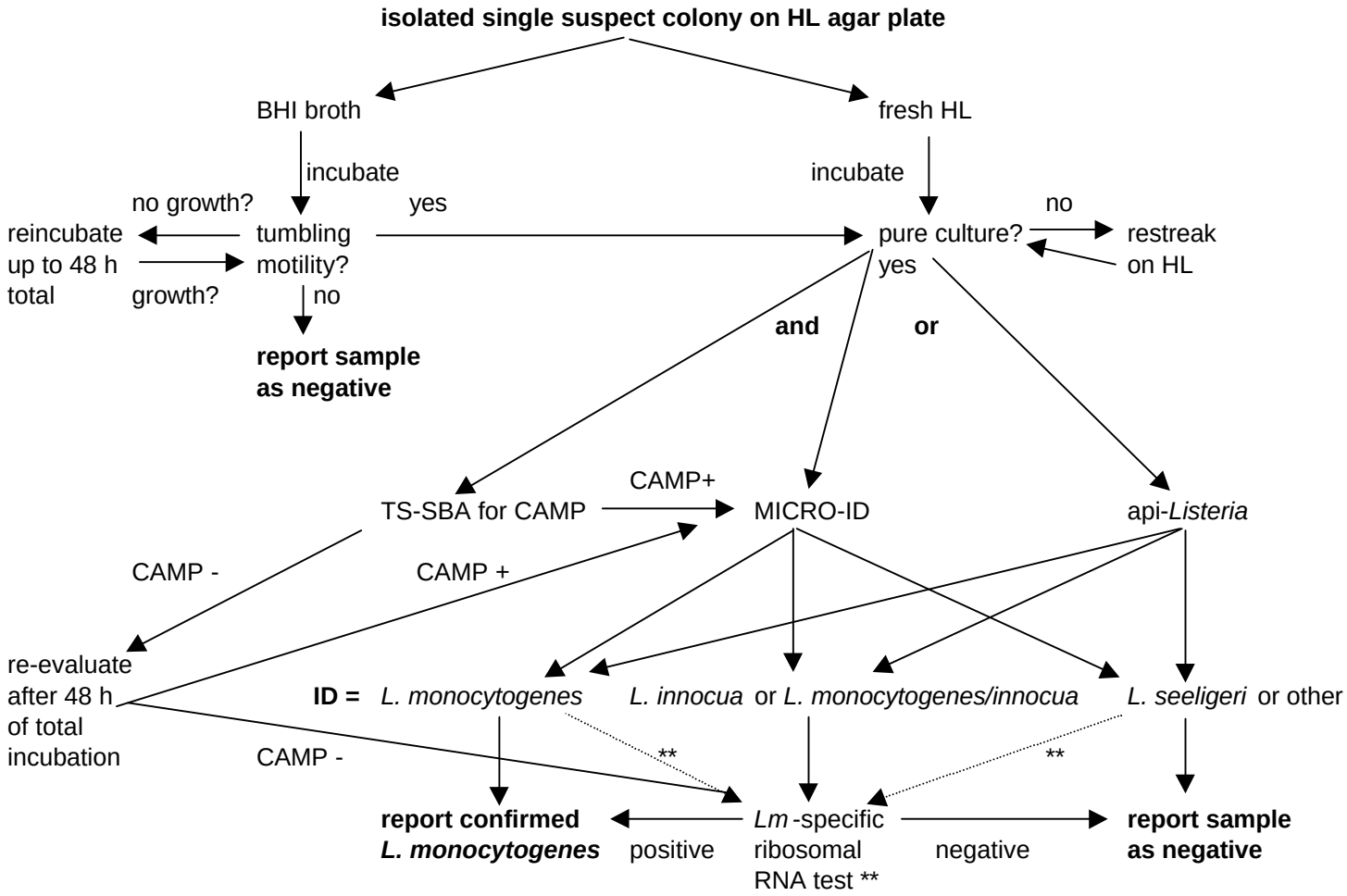


** Suspects must also be absent from other branches of analysis before the sample is considered negative.*

*** Confirmation generally requires 2-3 additional days.*

**** Requires 1-4 additional days of analysis.*

Diagram 2 **Flow diagram for confirmation of *Listeria monocytogenes*, MLG Chapter 8, Revision 2 (11/08/99)**



* The most likely time frame for completion of analysis.

** *L. monocytogenes*-specific ribosomal RNA-based test may optionally be applied to the confirmation of all isolates. It may be performed prior, concurrent or subsequent to biochemical and CAMP testing.

Listeria Media Appendix for Chapter 8, Revision 2 (11/08/99)

The ingredients and the chemicals used for preparing media for *Listeria monocytogenes* may be the product of any manufacturer if comparative tests show satisfactory results. The carbohydrates (sugars) should be chemically pure and suitable for biological use; inorganic chemicals used as reagents should be American Chemical Society (ACS) grade; and dyes must be certified by the "Biological Stain Commission" for use in media.

For convenience, dehydrated media or commercially prepared broths and plates of any brand equivalent to the formulation may be used. Be sure that the formulation of the commercially available medium is identical or has been appropriately validated as equivalent to the formulation specified below. Each batch of media should be tested for sterility and ability to support growth of positive and negative control organisms.

Hydrogen ion concentration (pH) of media should be determined using an electronic pH meter which is standardized against known buffers, prepared according to the Official Methods of Analysis of the Association of Official Analytical Chemists (16th Edition). If necessary the pH of a medium should be adjusted by adding sufficient 1 N sodium hydroxide or 1 N hydrochloric acid.

The percent solutions specified in this appendix are weight of solid to volume of diluent. For the dilute stock solutions specified in this appendix, a percent solution may be prepared by either of the following approaches (A 1% solution is used as an example):

a.) Dissolve 1 gram of solid into less than 100 ml of water, then qs to 100 ml in a volumetric flask.

b.) Dissolve 1 gram of solid into 100 ml of water.

Unless otherwise indicated, a medium should be sterilized by steam under pressure at 121°C (15 lb.) for 15 minutes.

BRAIN HEART INFUSION (BHI) AGAR

Calf Brain (infusion from)	200.0 g
Beef Heart (infusion from)	250.0 g
Proteose peptone or gelysate	10.0 g
NaCl	5.0 g
Na_2HP0_4	2.5 g
Dextrose	2.0 g
Agar	15.0 g
Distilled water	1.0 L

Dissolve ingredients in distilled water by heating to boiling. Dispense as desired and autoclave at 121°C for 15 minutes. Final pH 7.4 ± 0.2.

BRAIN HEART INFUSION (BHI) BROTH

Prepare same as above except omit the 15.0 g agar.

Dispense 5 ml in screw-capped tubes and autoclave at 121°C for 15 minutes. Final pH 7.4 ± 0.2.

DEY-ENGLEY NEUTRALIZING BROTH (D/E)

Tryptone	5.0 g
Yeast Extract	2.5 g
Dextrose	10.0 g
Sodium Thioglycollate	1.0 g
Sodium Thiosulfate	6.0 g
Sodium Bisulfite	2.5 g
Polysorbate 80	5.0 g
Lecithin (Soy Bean)	7.0 g
Brom Cresol Purple	0.02 g
Distilled water	1.0 L

Adjust to a final pH of 7.6 ± 0.2 at 25°C.

FRASER BROTH (FB)

This formulation contains 20 mg/L nalidixic acid and 25 mg/L acriflavine

Proteose Peptone	5.0	g
Tryptone	5.0	g
Lab Lemco Powder (Oxoid)	5.0	g
Yeast Extract	5.0	g
NaCl	20.0	g
KH_2PO_4	1.35	g
Na_2HPO_4	12.0	g
Esculin	1.0	g
Naladixic Acid (2% in 0.1 M NaOH)	1.0	ml
Lithium Chloride	3.0	g
Distilled water	1.0	L

Mix well to resuspend the media and dispense 10 ± 0.5 ml into tubes. Sterilize at 121°C for 15 minutes. DO NOT OVERHEAT; COOL AT ONCE AFTER REMOVAL FROM THE STERILIZER. Store in the refrigerator. Just before use, add 100 µl of 2.5 mg/ml of filter sterilized acriflavine (Sigma) and 0.1 ml filter sterilized 5% stock solution of ferric ammonium citrate (Sigma) in distilled water to each 10 ml tube.

The following alternatives may be employed:

a.) Rather than dispense acriflavine solution into individual tubes, 25 mg/L of acriflavine HCl may be incorporated into the medium prior to autoclaving.

b.) FB may be prepared from commercially available UVM1 by adding appropriate amounts of lithium chloride, acriflavine and ferric ammonium citrate.

FRASER BROTH MINUS FERRIC AMMONIUM CITRATE (FB-Fe)

Prepare Fraser Broth as described above but do not add ferric ammonium citrate solution.

HORSE BLOOD OVERLAY MEDIUM (HL, HBO)

Base Layer:

Columbia Blood Agar Base 1.0 L

Prepare according to manufacturer's specifications and sterilize at 121°C for 15 minutes. Pour 10 ml per 100 mm diameter Petri dish. Allow to solidify and while still warm, overlay with blood agar as described below.

Top Layer:

Add 4% sterile horse blood to a portion of melted Columbia Blood Agar Base which has been cooled to 46°C. Stir or swirl to mix evenly. Quickly place 5 or 6 ml on top of the base layer and tilt the plates to spread top layer evenly. Store plates in the refrigerator. Discard any plates which become discolored.

LISTERIA REPAIR BROTH (LRB)

a.) Add 0.30 g of $FeSO_4$ per liter of deionized water and allow to dissolve.
b.) Add and dissolve, while heating, the following components:

30.0 g/L Trypticase Soy Broth
5.0 g/L glucose
6.0 g/L yeast extract
8.5 g/L MOPS, free acid (4-morpholinepropanesulfonic acid)
13.7 g/L MOPS, sodium salt monohydrate (4-morpholinepropanesulfonic acid, sodium salt)

c.) After all the above components have dissolved completely, add 2.46 g/L $MgSO_4$.
d.) Allow the solution to come to a boil.
e.) Remove from heat and slowly add 10.0 g/L sodium pyruvate.
f.) Dispense into 225 ml aliquots and autoclave 15 minutes at 121°C/15 psi.
g.) Allow to cool before use.
h.) Following non-selective enrichment for 3-5 h, add the following antibiotics per 225 ml aliquot:

0.445 ml of 1.0% solution of Acriflavine
1.8 ml of 0.5% solution of Nalidixic Acid
1.15 ml of 1.0% solution of Cycloheximide (in 40% Ethanol)

MODIFIED OXFORD MEDIUM (MOX)

MOX Agar Base:

Columbia Blood Agar Base (depending on brand)	39-44.0-g
Agar	2.0 g
Esculin	1.0 g
Ferric Ammonium Citrate	0.5 g
Lithium Chloride (Sigma L0505)	15.0 g
1% Colistin Solution	1.0 ml
Distilled water	1.0 L

Rehydrate commercial Columbia Blood Agar Base with constant stirring using a magnetic mixer and adjust pH to 7.2, if necessary. Autoclave this base at 121°C for 10 minutes, mix again, and cool rapidly to 46°C in a water bath. Add 2 ml of 1% filter sterilized Moxalactam Solution to make the complete MOX medium, mix well, and pour 12 ml per plate.

CAUTION: If preparing MOX from a commercial premix, take care to select the correct antibiotic supplement.

1% Colistin Solution:

Colistin, Methane Sulfonate (Sigma C1511)	1.0 g
0.1 M Potassium Phosphate Buffer, pH 6.0	100.0 ml

Colistin solution is not sterile; store frozen in small aliquots (3-5 ml) at -20°C or below.

1% Moxalactam Solution:

Sodium (or Ammonium) Moxalactam (Sigma M1900)	1.0 g
0.1 M Potassium Phosphate Buffer, pH 6.0	100.0 ml

Dissolve, sterilize by filtration, dispense in 2 ml quantities and store in freezer at -20°C or below.

MODIFIED UVM BROTH (UVM, UVM1)

This formulation contains 20 mg/L nalidixic acid and 12 mg/L acriflavine.

Proteose Peptone	5.0 g
Tryptone	5.0 g
Lab Lemco Powder (Oxoid)	5.0 g
Yeast Extract	5.0 g
NaCl	20.0 g
KH_2PO_4	1.35 g
Na_2HPO_4	12.0 g
Esculin	1.0 g
Naladixic Acid (2% in 0.1 M NaOH)	1.0 ml
Acriflavine HCl	12.0 mg
Distilled water	1.0 L

Sterilize at 121°C for 15 minutes. DO NOT OVERHEAT; COOL AT ONCE AFTER REMOVAL FROM THE STERILIZER. IF THE MEDIUM BLACKENS OR DARKENS, IT HAS BEEN OVERHEATED AND MUST BE DISCARDED. Store in the refrigerator.

TRYPTICASE SOY AGAR-YEAST EXTRACT (TSA-YE)

Trypticase	15.0 g
Phytone	5.0 g
Sodium Chloride	5.0 g
Yeast Extract	6.0 g
Agar	15.0 g
Distilled water	1.0 L

Suspend the above ingredients in distilled water and dissolve completely by heating to boiling while stirring. Autoclave for 15 minutes at 121°C. Temper the medium to 45 - 50°C and pour into sterile Petri dishes. Alternatively, TSA-YE may be prepared as slants for short term storage of *Listeria* spp.

TRYPTICASE SOY-SHEEP BLOOD AGAR (TS-SBA, CAMP agar)

Trypticase	15.0 g
Phytone	5.0 g
Sodium Chloride	5.0 g
Agar	15.0 g
Distilled Water	1.0 L

Suspend the above ingredients in distilled water and dissolve completely by heating to boiling while stirring. Autoclave for 15 minutes at 121°C. Cool to ca. 50°C, then add 50 ml of tempered sterile defibrinated sheep blood and swirl gently until evenly distributed in the medium. Avoid bubble formation. Dispense 8-15 ml into each petri dish for solidification. To facilitate interpretation of the CAMP test, 8-10 ml (i.e. “shallow”) plates are recommended.